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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The mortality and morbidity of prostate cancer (CaP), the commonest cancer in men in Western society, are largely caused by the spread of CaP to bone. How this occurs, and the interactions between CaP cells and bone are poorly understood. Specific mutations of the tumor suppressor gene, *p53*, that occur in CaP may cause disease progression. To test this, we have generated cell lines derived from the human LNCaP cell line that stably express wild type or mutant *p53*.

Purpose: Using generated lines, to test whether *p53* mutations affect establishment and growth of experimentally-induced CaP in the bone.

Scope: Our initial studies in tissue culture will show that factors produced by the variant LNCaP lines may alter the normal processes of bone remodeling and angiogenesis.

Results/Progress: No effects were seen on the proliferation of bone forming cells; those on cellular differentiation are currently under study. We have perfected the technique for implanting CaP cells in the bone of immunologically deficient mice, and established a "take rate" for some LNCaP lines.

Significance: Further studies will provide information about how specific mutations of *p53* found in patients with clinical disease impact on progression, and could lead to the development of new therapeutic strategies.

14. SUBJECT TERMS:
preclinical studies, cellular interactions, p53 mutations, stably transfected LNCaP lines, osteoblast proliferation and differentiation

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THE ROLE OF p53 MUTATIONS IN METASTASIS OF PROSTATE CANCER TO BONE

Annual Report, DAMD17-02-1-0108

INTRODUCTION:

The mortality and morbidity of prostate cancer (CaP), the most common cancer in men in Western society, are largely due to bony metastases, yet how this occurs, and the cellular interactions between CaP cells and the bone microenvironment, are poorly understood. The **subject** of this work is to study the role of *p53* mutations in the metastasis of prostate cancer to bone. We have generated a series of cell lines derived from the prostate cancer cell line, LNCaP, that stably expresses wild type *p53* (Wt) or *p53* mutants (F134L, M237L and "hotspot" R273H), detected in different clinical prostate cancers. For comparison, we also have the Empty line, stably transfected with the plasmid vector, pRcCMV2 and the untransfected parent line. Based on the hypothesis that specific *p53* mutations found in prostate cancers are involved in promoting tumor progression of CaP, our **purpose** is to use these lines to test the role of *p53* mutations in the establishment and growth of experimentally-induced CaP bone metastases. The **scope** of research involves initial studies performed *in vitro* to determine the effects of factors produced by the LNCaP parent and transfectant lines on the normal process of bone remodelling and on angiogenesis. Stable LNCaP transfectants expressing Wt or mutant *p53* will then be implanted in the tibia to assess their potential to form osteoblastic lesions, and orthotopically in the prostate of SCID mice, to determine their ability to spontaneously metastasize to bone. This work will provide information about how specific mutations of *p53* found in patients with clinical disease impact on disease progression, and could identify targets for further study.

BODY:

For each of the studies described below, 6 LNCaP cell lines were used. These were: LNCaP-parental, LNCaP-Empty, LNCaP-Wtp53 (overexpresses Wt p53), LNCaP-F134L (expresses mutant p53), LNCaP-M237L (expresses mutant p53) and LNCaP-R273H (expresses "hot-spot" mutant p53); for convenience, these have been abbreviated as LNCaP, Empty, Wt, F134L, M237L and R273H, respectively. In the first year, we aimed to accomplish Tasks 1A, 1B, 1C, 2A and 3A, and to begin Tasks 1D, 2B and 3B. After the grant had been accepted, it took some time before we were able to employ suitable staff to carry out the work. Elizabeth Kingsley* has been involved in producing conditioned medium, examining its effects on osteoblast cells by RT-PCR and protein analysis; Barbara Szymanska*, PhD pending, joined us in late June 2002, and was involved in work on mineralization of collagen, and studies of osteoblast and osteoclast differentiation; Lara Perryman*, was appointed on the 1st of May 2002, and was involved in cell culture, injecting of mice and analysis of procedures. Julie Brown* initiated the osteoblast studies and the bone injection and has helped with each of these tasks. (* These staff have salaries from the grant; Dr Brown has only 20% of her salary from this grant).We have accomplished all of Task 1A, but decided to expand some the data sets obtained (see below); Tasks 1B, 1C and 2A have been initiated and problems that were encountered have all been resolved, and the work is now continuing apace, given that we are now fully staffed. Tasks 1D, 2B, 3A and 3B were also initiated, as anticipated. In addition, we have initiated experiments for Task 1E, programmed for months 13-18. The following report details the results obtained in each of these sections.

TASK 1A: Determine the effects of transfected LNCaP cells on osteoblast proliferation *in vitro*

Objective:

Three osteosarcoma cell lines, U-2 OS, MG-63 and Saos-2, which mimic osteoblasts at various stages of differentiation, were treated with conditioned medium (CM) produced by the six p53-variant LNCaP prostate cancer cell lines (described above), in order to determine any effects upon cell proliferation.

Methods:

i. Production of CM: CM was produced from the six LNCaP lines, LNCaP, Empty, Wt, F134L, M237L, R237H. Cells were grown to ~70% confluency in 150 cm^2 flasks, then washed twice with phosphate buffered saline, pH 7.2 (PBS), and fed 25 mL of serum-free (SF) T-medium (see Appendix 1). After 24 hours, the CM were collected, centrifuged to remove any cellular debris, aliquotted, and stored at -20°C. This method was used for the production of all CM (see below).

ii. Determination of optimal seeding for osteosarcoma cell lines: The appropriate seeding numbers for the three osteosarcoma cell lines were determined by carrying out cell density experiments. Cells were seeded into 96-well plates, in 100 µL/well, at 100, 500, 1000, 2500, 5000 and 10000 cells per well. The cells were fed with 100 µL of fresh medium on the next two days, in order to mimic experimental conditions (see below). On the third day, the cells were examined under the microscope to select a seeding number for each line which would give a confluency of ~ 70% on the third day; this was so any proliferative effect of the conditioned media could be detected. For the U-2 OS line, 1000 cells/well were chosen; for the MG-63 and Saos-2 lines, 2000 cells/well.

iii. Proliferation assays, osteosarcoma cell lines: Proliferation assays were carried out in quadruplicate for each of the three osteosarcoma lines, in the presence of all six CM. Cells were seeded into 96-well plates at the appropriate density on Day 0. On Day 1, the cells were fed with media solutions containing various concentrations of CM. As LNCaP cells grow in T-medium, and the osteosarcoma lines in DMEM, the conditioned media solutions were adjusted so that all cells were exposed to a similar concentration of the T-medium supplements. Fetal bovine serum (FBS) was added to the standard 10% concentration. The solutions used were as follows:

0% CM (per mL):	100 µL FBS + 400 µL SF DMEM + [500 µL SF T-medium]
5% CM (per mL):	100 µL FBS + 400 µL SF DMEM + [450 µL SF T-medium + 50 µL CM]
10% CM (per mL):	100 µL FBS + 400 µL SF DMEM + [400 µL SF T-medium + 100 µL CM]
25% CM (per mL):	100 µL FBS + 400 µL SF DMEM + [250 µL SF T-medium + 250 µL CM]
50% CM (per mL):	100 µL FBS + 400 µL SF DMEM + [500 µL CM]

On Day 2, the cells were re-fed with freshly prepared CM solutions. On Day 3, the media were replaced with phenol red-free, serum-free DMEM, and cell proliferation assessed using the WST-1 Cell Proliferation Assay (Roche; Cat. No. 1 644 807), as per the manufacturer's instructions. Optical density readings were taken at 450 nm, and the data analysed using one-way ANOVA at a confidence interval of 95%.

Results:
No statistically significant differences in proliferation were observed for any of the three osteosarcoma cell lines following treatment with any of the CMs from the six LNCaP lines. However, some effects were recorded, most noticeably an increase in both U-2 OS and MG-63 proliferation following treatment with 10% F134L CM; and a decrease in MG-63 proliferation following treatment with 50% R273H CM. It was decided to repeat these experiments, extending the treatment of the cells with the CM to three, five and seven days. These experiments are currently underway. Thus the initial experiments were completed, but as we obtained some trends that were not statistically significant, we decided to increase the time of exposure of the osteoblast cell lines to CM, to determine whether the effects would become more marked. Whilst this part of the work was outside of the scope of the nominated Task 1A, we believe that additional work could provide productive data.

TASK 1B: Determine the effects of transfected LNCaP cells on osteoblast differentiation *in vitro*

TASK 1C: Effects of LNCaP transfectants on collagen production

(**NB:** As the early stages of the work for these two tasks were identical, all experiments were conducted simultaneously).

Objectives:

To isolate RNA and protein from homogenates of osteosarcoma cell lines treated with CM from LNCaP parent and transfected lines: The three osteosarcoma cell lines were treated with the CM from the six LNCaP lines for various lengths of time. The cells were harvested and counted, then homogenized in TRI Reagent (Sigma; Cat. No. T-9424). Currently, RNA is being extracted from these homogenates, and the cDNA analysed by reverse-transcriptase polymerase chain reaction (RT-PCR) in order to determine the effects of CM treatment upon the expression of bone markers including alkaline phosphatase (ALP), osteocalcin (OCN), type I collagen (COL) and osteoprotegerin (OPG). Alkaline phosphatase and osteocalcin are early- and late-stage markers of osteoblast differentiation, respectively. Type I collagen is produced by osteoblasts during the formation of new bone, while osteoprotegerin is a bone resorption inhibitor that blocks the formation and activation of osteoclasts.

Protein will also be extracted from the homogenates, and used for Western blotting.

Prior to the harvesting of the treated cells, samples were taken of all culture supernatants (SNs). These will be analysed for the presence of secreted factors using various EIA kits.

Task 1B-a/Task 1C-a, c: Osteoblast differentiation assays

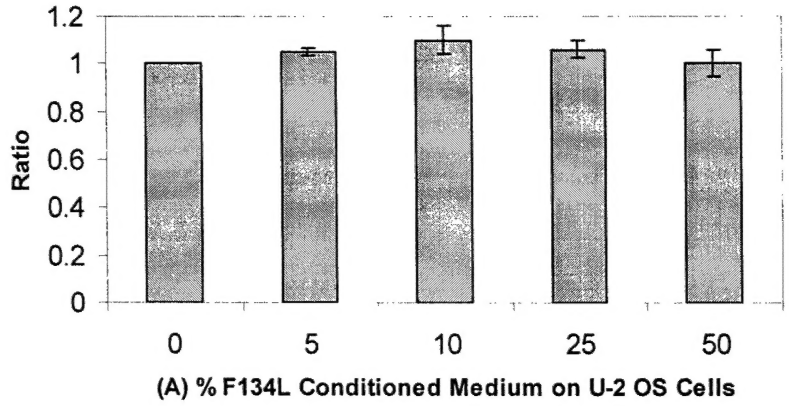
These experiments were carried out in triplicate for all three osteosarcoma cell lines exposed to CM from the six LNCaP lines - i.e. 54 experiments in total.

Methods:

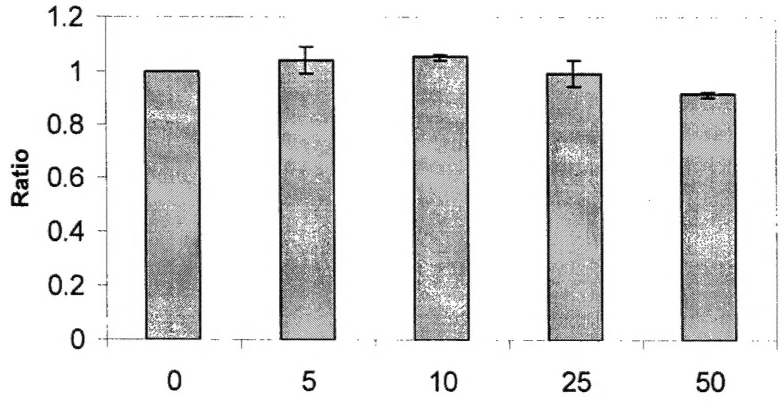
i Preparation of CM: (see i above) In order to save both time and effort, it was decided to undertake large-scale production of CM, and produce all six media in the quantities required to perform **Tasks 1B**, **1C**, and **1D** and **Task 2B**. This work was carried out in parallel using

the method described above (see **Task 1A,** Method i). In total, >1000 mL of CM from each the six LNCaP cell lines were produced, aliquotted, and stored at -80°C.

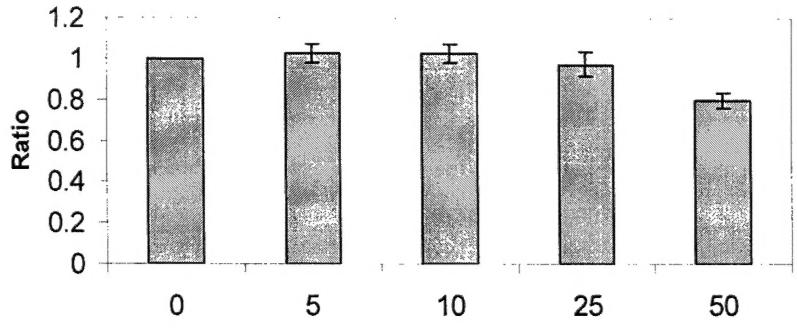
FIGURE 1: Effects of CM on proliferation of osteosarcoma cells *in vitro*.



(A) % F134L Conditioned Medium on U-2 OS Cells



(B) % F134L Conditioned Medium on MG-63 Cells



(C) % R273H Conditioned Medium on MG-63 Cells

Figure 1: Results of proliferation assays. Osteosarcoma cells were treated for 48 hours with various concentrations of conditioned medium (CM) produced from the six LNCaP cell lines. Cell proliferation was determined using the WST-1 system (see text). Results were expressed as a ratio of the control (0% CM). (A) U-2 OS cells treated with F134L CM; (B) MG-63 cells treated with F134L CM; (C) MG-63 cells treated with R273H CM.

iv. Differentiation of osteoblasts: Assays were carried out as follows:

Day 0:

The U-2 OS and MG-63 osteosarcoma cell lines require 3.0×10^7 cells per experiment; the Saos-2 cell line, 3.75×10^7 cells per experiment.

U-2 OS or MG-63 cells were seeded into 25 x 75 cm^2 flasks in 10 mL medium, at a seeding density of 1.2×10^6 cells per flask (i.e. ~ 1.6×10^4 cells per cm^2). This is a density calculated from previous studies by Julie Brown from her work with these cell lines, as encouraging differentiation over proliferation.

Saos-2 cells were more difficult to handle. Experience indicated that there tended to be cell loss when this line was seeded. Furthermore, trypsinization often needed to be carried out over an extended time period (over an hour, compared with 10-20 minutes for U-2 OS and MG-63), and even then cell removal was sometimes incomplete. For these reasons, it was decided to seed the Saos-2 cells into 100 mm dishes rather than 75 cm^2 flasks, and to harvest them by scraping rather than by trypsinization. In addition, due both to the loss of cells during seeding and the slow doubling times of the cell line, the Saos-2 cells were seeded at a higher density: 1.5×10^6 cells per 100 mm dish (i.e. ~ 2.5×10^4 cells per cm^2).

Day 1:

The cells were treated with 10 mL medium containing various concentrations of CM. The FBS content was reduced to 2%, to minimize the impact of early response genes on regulating ALP, OCN and COL expression.

The appropriate number of CM aliquots were thawed, pooled, and filtered through 0.2 μm filters to ensure sterility. The CM treatment solutions were prepared as follows:

0% CM (per 50 mL):	1 mL FBS + 24 mL SF DMEM + [25 mL SF T-medium]
5% CM (per 50 mL):	1 mL FBS + 24 mL SF DMEM + [22.5 mL SF T-medium + 2.5 mL CM]
10% CM (per 50 mL):	1 mL FBS + 24 mL SF DMEM + [20 mL SF T-medium + 5 mL CM]
25% CM (per 50 mL):	1 mL FBS + 24 mL SF DMEM + [12.5 mL SF T-medium + 12.5 mL CM]
50% CM (per 50 mL):	1 mL FBS + 24 mL SF DMEM + [50 mL CM]

For each individual experiment, the medium was aspirated from all 25 flasks/dishes, and the cells washed once with 5 mL PBS; the PBS was also aspirated. The cells were then fed with the CM solutions, with 5 flasks/dishes per CM concentration

At the appropriate timepoints (3, 6 and 12 hours after initial exposure), 1 flask/dish of each CM concentration was harvested (5 flasks/dishes per timepoint). A 1 mL sample was taken of the culture supernatant (SN) of each flask/dish for the analysis of secreted factors; these were stored at -20°C. The remaining SN was then aspirated, the cells washed once in 5 mL PBS, then harvested by either trypsinization or scraping. The cell suspensions were counted, then centrifuged. The SNs were aspirated, and the cell pellets homogenized in TRI Reagent (Sigma; Cat. No. T-9424) as per the manufacturer's instructions. These samples were stored at -80°C, until processed for the extraction of RNA and protein.

Day 2:

The 24 hour timepoint flasks/dishes were harvested and processed as described above. The 48 hour timepoint cells were re-fed with a further 10 mL of the appropriate, freshly prepared CM solutions.

Day 3:

The 48 hour timepoint flasks/dishes were harvested and processed as described above.

Initially, it was planned to conduct these experiments in duplicate for each cell line (i.e. 36 experiments); process the samples to extract RNA and protein; produce cDNA; and carry out PCR analysis. However, during the latter part of the year, negotiations for a collaboration between this research group and the Corbett Research Australia, Invitrogen company were initiated. Should this collaboration come to pass, it would allow the analysis of the samples to be carried out in a far more rapid and cost-efficient manner. In view of this, it was therefore decided to put off the analysis of the samples, and to complete the triplicate experiments instead.

These experiments have now been completed, giving a total of 1350 samples each of cell homogenate in TRI Reagent, and of culture supernatant.

v. Production of cDNA from RNA and protein extraction: RNA extraction, protein extraction (for Western blotting, TASK 1B-e), and cDNA production of the experimental samples is currently underway.

Task 1B-a/Task 1C-b: Reverse transcriptase polymerase chain reaction

vi. Design of primers and optimization of RT-PCR conditions:
Primers were designed and obtained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; positive control), alkaline phosphatase (ALP), osteocalcin (OCN) and type I collagen (COL) (Table 1). Reverse transcriptase polymerase chain reaction (RT-PCR) conditions for these primers using *Taq* polymerase were optimized using MG-63 cDNA produced from RNA extracted from TRI Reagent homogenates. Initially, annealing temperature and $MgCl_2$ conditions for all four primer sets were optimised using a 48-sample "checkerboard" system in a Hybaid Multistep Gradient PCR block, with 30 cycles chosen arbitrarily. $MgCl_2$ concentrations of 1.5, 2.0, 2.5 and 3.0 mM and a temperature gradient spanning 57°C - 72°C, with twelve "steps", were assessed.

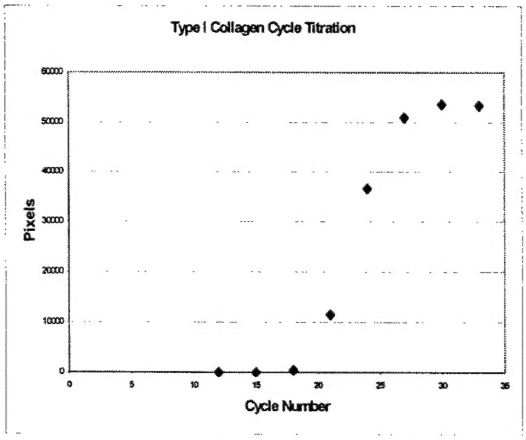
For each primer set, the 48 PCR products were run on 1% agarose gels containing 0.2 μg/mL ethidium bromide, at 100 V for 30 min. The gels were visualized under UV light, and photographed. The results were examined, and the best combination of $MgCl_2$ and temperature chosen for each primer set. Using these conditions, cycle number was then titrated for each set, with a sample being removed from the PCR block after 12, 15, 18, 21, 24, 27, 30, 33 and 36 cycles (i.e. 9 samples per primer set). The appropriate amount of cDNA (1 or 2 μL) to use with each set was also assessed. These sample sets were also examined by agarose gel electrophoresis as described. Using the Electrophoresis Documentation And Analysis System (EDAS; Kodak), densitometry was performed upon each gel, and the band intensities graphed against cycle number. From these graphs, the most appropriate cycle

number (i.e. within the log phase of the graph) was chosen for each primer set (Figure 2, Figure 3, see Appendix 2).

Table 1: Optimized RT-PCR conditions

Primer set	Sequences	µL cDNA	$MgCl_2$ Concentration	Annealing Temperature	Cycle Number
GAPDH	Sense: CCACCCATggCAAATTCCATggCA Antisense: TCTAgACggCAggTCAggTCCACC	1 µL	1.5 mM	60°C	21
ALP	Sense: CCAgAgAAAgAgAAAgACCCCAAgTA Antisense: ATgCCCACAgATTTCCCAgCgTCCTT	2 µL	1.5 mM	65.4°C	30
COL	Sense: AAgACgACATCCCACCAATCAC Antisense: AgCTTCCCCATCATCTCCATTCTTT	1 µL	1.5 mM	61.5°C	22
OCN	Sense: CCCTCACACTCCTCgCCCTATTggCCC Antisense: gggCAAggggAAgAggAAAgAAgggTgC	2 µL	2.5 mM	71°C	28

Figure 2: Cycle titration using primers for type I collagen



Result of a representative cycle titration, using the COL primer set with *Taq* polymerase and 1.5 mM $MgCl_2$, at 61.5°C. Band intensity (pixels) was graphed against the cycle number. Twenty-two cycles was chosen as the optimal cycle number, since this was in the middle of the log phase.

While the GAPDH, ALP and COL primer sets were successfully optimized, some problems were encountered with the OCN primers, which amplified a number of spurious bands. Amplification of RNA rather than cDNA with the OCN primers confirmed that this was due to the presence of contaminating genomic DNA. The OCN RNA and genomic DNA sequences are similar in size; "intron-jumping" does not induce sufficient differences to prevent the amplification of the latter.

Two alternative procedures were investigated. Firstly, the RNA itself was treated by a variety of methods, in order to remove or destroy the genomic DNA. These were:

- additional phenol/chloroform extraction steps during RNA extraction
- UV irradiation
- RNase-Free DNase (Promega; Cat. No. M6 101)

- DNA-*free* (Ambion; Cat. No. 1906)

Use of DNA-*free* according to the manufacturer's instructions succeeded in removing the contaminating genomic DNA from the RNA samples; none of the other methods was successful.

At this time, a variety of PCR "mastermixes" were made available, and trialed against each other using the OCN primers. These were:

- ReddyMix (ABgene; Cat. No. AB-0575/DC/LD)
- PCR Supermix (Invitrogen; Cat. No. 10572-014)
- Platinum PCR Supermix (Invitrogen; Cat. No. 11306-016)

Initial results with these mixes were very encouraging. All three systems gave much higher amplification of the specific band, with the spurious bands being less obvious. Further optimization experiments, reducing the annealing and extension times from 30 seconds to 20 seconds, and 90 seconds to 30 seconds, respectively, improved the results even more. Under these conditions, the Platinum PCR Supermix gave excellent results, with a strong OCN band present, the spurious bands eliminated, and some "primer dimer" activity significantly reduced. (Figure 4, see Appendix 3). Further "fine-tuning" of this system with the OCN primers is currently underway.

vii. Sequencing of the PCR products:

The ALP, OCN and COL PCR products were sequenced, in order to confirm that the primers sets were amplifying the correct sequences. Five μL of COL product, and 15 μL each of the OCN and ALP products, were run on a 2% agarose gel containing ethidium bromide at 0.1 μg/mL at 70 V for 30 min, then at 80 V for a further 30 min. The bands were visualized under UV light, and the specific bands cut from the gel with a scalpel blade. Each gel slice was transferred into a pre-weighed Eppendorf tube, and the DNA purified from the agarose using the GeneClean III kit (Bio101, Carlsabad, CA; Cat. # 1001-600) as per the manufacturer's instructions.

The purified fragments were ligated into vectors using the pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen, Carlsbad, CA; Cat. # K4900-40), which allows direct insertion of *Taq* polymerase-amplified PCR products, as per the manufacturer's instructions. "TOP 10" *E. coli.* bacteria (transformation efficiency >1 x 10^9 cfu/μg DNA) were transformed with 2 μL of each ligation reaction (i.e. three reactions), then 250 μL SOC medium (see Appendix) were added to each vial, and the suspensions incubated for 1 hour at 37°C with shaking. The bacteria were plated out on agar containing 0.1 mg/mL ampicillin (50 or 200 μL/plate), and incubated inverted for 16 hours at 37°C. The colonies were expanded in Circlegrow medium (40 g/L; Bio101, Carlsbad, CA; Cat. # 3000-122) containing 0.1 mg/mL ampicillin for 16 hours at 37°C with shaking. The plasmid DNA was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI; Cat. # A1460) as per the manufacturer's instructions. DNA concentrations were determined by spectrophotometry at 260/280 nm.

Restriction enzyme digests were then carried out in order to confirm that the plasmid preparations contained insert. Examination of the restriction map supplied with the TOPO vector indicated that the inserts could be cut out using *Hind*III and *Eco*321. The sequences for ALP, COL and OCN were obtained using the Genbank program. Restriction enzyme

mapping indicated that OCN could be cut with *Bam*H1 and COL with *Kpn*1, but no suitable restriction site was present within the ALP insert. Therefore, triple digests were carried out for OCN and COL, but a double digest for ALP. Ten μL of each plasmid preparation were digested for 4 hours at 37°C. The digests were run on 2% agarose gels containing 0.1 μg/mL ethidium bromide at 80 V for 1 hour, and examined under UV light. All three digests gave the expected band sizes (including a 68 bp overhang produced by the *Hind*III/*Eco*321 digest of the vector).

Sequencing of the three inserts was then carried out at the University of Sydney. The results of this sequencing were compared to the consensus sequences for all three products. All three primer sets were determined to be amplifying the correct product.

TASK 1B-b, -c, and -d. Quantification of ALP, OCN, COL and OPG levels secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

TASK 1C-d. Assay for carboxyterminal propeptide of type I collagen using the Prolagen-C kit secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

Analysis of the secreted factors has been delayed due to difficulties experienced in obtaining the necessary kits.

(1) Metra™ *CICP EIA kit* (Quidel Corporation, San Diego, CA; Cat. # 8003) - for the detection of the C-terminal propeptide of type 1 collagen in serum/culture supernatant
(2) Intact human osteocalcin ELISA kit (Biomedical Technologies, Stoughton, MA; Cat. # BT-460)
(3) Osteoprotegerin enzyme immunoassay (Biomedica, Gesellschaft, mbH; Cat. # BI-20402)
(4) Alkaline phosphatase diagnostic kit (Sigma, St Louis, MO; Cat. # 104-LL)

In one case, a new company took over the local distribution of one of the required kits, but was unable to acquire the necessary AQIS (Australian Quarantine and Import Services) permits to import them for over five months. However, all kits have now been obtained, and analysis of the SN samples is underway.

Task 1D. Effects of LNCaP transfectants on mineralization of collagen

Objectives:

The overall aim was to investigate the effect of factors secreted by the six LNCaP cell lines on matrix mineralization by human osteoblastic cell lines, Saos-2 and U-2 OS. However, we have shown that U-2 OS cells do not produce mineralized matrix *in vitro*.

Objective 1: To establish a method for quantitative analysis of mineral deposited by the osteoblastic cell lines, U-2 OS, Saos-2 and in addition, a mouse calvarial cell line, MC3T3-E1, that was used as a positive control in this study. MC3T3-E1 cells have been widely used to study *in vitro* matrix mineralization (Sudo *et al.*, 1983, Kodama *et al.*, 1986, Fratzl-Zelman *et al.*, 1998).

Methods:

viii. Visualization of mineralised extracellular matrix: Mineralized extracellular matrix is commonly visualized by either a von Kossa (Rungby *et al.*, 1993) or Alizarin red S (Hale *et al.*, 2000) staining of calcium phosphate deposits. The two methods of staining were compared using densitometry and Alizarin red S staining was established as the method of choice for these experiments. The staining protocol was optimized for the Saos-2 and MC3T3-E1 cell lines as follows: Saos-2 cells were stained with 1% Alizarin red S for 1 minute and MC3T3-E1 cells were stained with 2% Alizarin red S for 1 minute.

ix. Optimization of methods: Effects of glucose on matrix mineralization:
Balint *et al.* (2001), have suggested that high glucose concentration (15 mM) in the medium inhibits matrix mineralization of mouse calvarial cell line MC3T3-E1 *in vitro*. We have conducted a series of experiments designed to investigate the effects of glucose concentration on matrix mineralization and proliferation of Saos-2 and U-2 OS cells, as both of these cell lines are maintained in high glucose (25 mM) medium in our laboratory. In our hands, there was no difference between the quantity of mineralized matrix produced by Saos-2 cells maintained in low versus high glucose medium. However, glucose concentration appeared to have an effect on Saos-2 cell proliferation: the cells proliferated faster in the presence of higher glucose concentration. In contrast, glucose concentration did not appear to affect proliferation of the U-2 OS cells and it did not influence the ability of these cells to produce mineralized matrix. The effect of high glucose concentration on MC3T3-E1 cell matrix mineralization/ proliferation was not investigated as these cells are maintained in low glucose (10 mM) medium.

x. Further optimization of methods: In an attempt to upregulate the production of mineralized matrix the effects of two osteogenic factors, dexamethasone and calcitriol (active vitamin D metabolite) on matrix mineralization of Saos-2 and MC3T3-E1 cells was also examined. It was found that 10^{-8} M calcitriol completely abolished the MC3T3-E1 matrix mineralization. In contrast, dexamethasone had a small upregulatory effect on matrix mineralization, which was maximal at 10^{-7} M. Preliminary data suggests that calcitriol also appears to inhibit and dexamethasone increase the production of mineralized matrix in Saos-2 cells.

xi. Optimized method for studying matrix mineralization by MC3T3-E1 cells *in vitro*: The mouse calvarial cell line MC3T3-E1 was seeded at 5 x 10^4 cells/ well in a 6 well plate in αMEM 10% FBS and cultured for 7 days, after which 10 mM β-glycerophosphate and 10^{-7} M dexamethasone were added to the culture medium in order to induce matrix mineralization. The cells were incubated for two weeks with medium changes every 2-3 days. Following the incubation period, the medium was removed, the cell layers were washed twice with PBS and fixed in 10% neutral-buffered formalin for 30 minutes at room temperature. The cells were then washed twice in distilled water and stained with 2% Alizarin red S for 1 minute. The stain was then removed and the cell layers washed twice with distilled water and allowed to air-dry. The results were analysed by densitometry using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Results:

The method for quantitative analysis of mineral deposited by the MC3T3-E1 cells has been optimized (Figure 5, see appendix 4). The optimization of culture conditions for studying matrix mineralization of Saos-2 cells is in progress and will soon be completed.

TASK 1D-a To treat osteoblastic cultures with varying concentrations of CM from the six LNCaP lines to determine their effects on matrix mineralization by osteoblastic cell lines.

It was originally proposed that two osteoblastic cell lines, Saos-2 and U-2 OS, would be used in this study. The U-2 OS cells did **not** produce mineralized matrix under the conditions tested. Therefore, we have included the MC3T3-E1 cell line, in addition to the Saos-2 cells, in this study. In order to investigate the effects of factors secreted by the six different LNCaP parent and transfectant lines, the MC3T3-E1 cells will be exposed to varying concentrations of the CM that has already been prepared as described in Task 1A (Method i).

Preliminary work: LNCaP cells are grown in T medium, which is based on DMEM (see Appendix 1), but we have shown that DMEM does not adequately support MC3T3-E1 cell growth and as a result, cell death occurs. It was therefore necessary, prior to commencing experiments using CM, to establish whether the two week's exposure of MC3T3-E1 cells to 50% T medium would allow them to produce mineralized matrix. It was also necessary to establish the minimal concentration of FBS required for matrix mineralization by these cells. We have established that the MC3T3-E1 cells can produce mineralized matrix in the presence of 50 % T medium and 5 % FBS and these conditions will be used in the studies to follow, which will commence in February 2003. The work with Saos-2 cells will commence in March following conformation of the preliminary data collected to date.

Task 1E. Effects of CM from LNCaP parent and transfected lines on mouse osteoclast differentiation *in vitro*.

The aim was to investigate the effect of factors secreted by the six LNCaP cell lines (parent, Empty, and those expressing either Wt or mutant p53) on osteoclastic bone resorption.

Task 1E-a. Growth of human or mouse osteoclasts from bone marrow cells

Methods:

xii. Isolation of bone marrow cells from mouse femur: The following method of bone marrow extraction and purification has been developed. C57BL/ 6J male mice were sacrificed using an overdose of ethrane. The fur and the muscle on the leg were removed to expose the femur. The epiphyses on both sides of the femur were removed and the bone marrow flushed out with 5 ml of Hanks' Balanced Salt Solution using a syringe and a 25G needle. The collected cells were transported on ice into the tissue culture facility where the cells were strained through a 100 μm nylon strainer to remove muscle and bone debris. The cells were then pelleted and the red blood cells lysed using 0.16M ammonium chloride (NH_4Cl) solution. The remaining white blood cells were washed and resuspended in αMEM with 10% FBS and plated onto dentine discs placed in a 96 well plate. The cells were allowed to attach overnight after which unattached cells were removed.

xiii. Differentiation of osteoclasts from precursor cells: It was necessary to establish a protocol to stimulate differentiation of osteoclasts from osteoclast precursors, to optimize conditions ensuring bone resorption by mature osteoclasts, and to develop a method for the quantitative assessment of osteoclast-bone resorptive activity (resorption pits).

Osteoclast differentiation from precursor cells has been reported to require stimulation with two factors: receptor activator of nuclear factor-κB ligand (RANKL) (Hofbauer and

Heufelder, 2001) and colony-stimulating factor-1 (CSF-1) (Scheven, Milne and Robins, 1997). In these reported studies, stimulation of mouse bone marrow cultures with 30 ng/ml RANKL and 25 ng/ml M-CSF for 3-4 weeks resulted in the appearance of cells staining positively for tartrate-resistant acid phosphatase, indicating the presence of osteoclasts. However, in our hands, these culture conditions required further optimization as the cells appeared to be relatively small compared with active bone-resorbing osteoclasts. To overcome these problems, we have contacted Julian M. W. Quinn from St. Vincent's Institute of Medical Research, Victoria, Australia, who is an expert in the field of osteoclast biology. We are seeking his advice regarding osteoclast culture and analysis of pits and exploring the possibility of establishing an ongoing collaboration with him.

TASK 2A Effects of LNCaP transfectants on endothelial cells proliferation *in vitro.*

Objective:

The human endothelial cell line, HUV-EC-C, will be treated with CM produced by the six LNCaP cell lines (parent, Empty, and those overexpressing Wt or expressing mutant p53) to determine whether there are any effects on cell proliferation.

Methods:

TASK 2A-a

i. Production of CM: This has been completed and was done as described above (Task 1A, method i).

TASK 2A-b

xiv. Creating stocks of the HUV-EC-C cells: An ampoule (1 x 10^6 cells) of the human endothelial cell line, HUV-EC-C (CRL-1730, ATCC, and passage 15) did not arrive until late June 2002. When initially defrosted these cells took 2 weeks to reach 70% confluency. This was much longer than expected given a reported doubling time of 31 hours (ATCC), although later passages grew more rapidly. Stocks were grown and frozen for the following 3 months. We did not proceed with experiments until 20 ampoules had been stored (passages 16 to 23), and then continued to freeze more stocks. Currently there are thirty ampoules ($5x10^5$-$1x10^6$ cells/ampoule).

xv. Determination of optimal seeding for HUV-EC-C cells: The appropriate seeding numbers for HUV-EC-C cells to ensure that they were in log phase after 1 week in culture were determined by carrying out cell density experiments. Cells were seeded, in 100µL, in triplicate in 96 well plates at 0, 250, 500, 1000, and increments up to 32000/well. Every 2-3 days, cells were fed with 100µl of fresh F12K medium, 10% FBS, with additives (HUV-EC-C medium, Appendix 1) and examined for growth. On day 7, the media were replaced with phenol red-free, serum-free DMEM and cells in 100µL of this medium were seeded for the standards in doubling dilutions from 256000 to 100/well; the control contained no cells. After adding 10µl of the WST-1 Cell Proliferation Assay (Roche; Cat# 1 644 807) reagent to each well, the optical density was read after 0.5, 1, 2, 4 and 8 hours at 450nm (Sunrise Tanch Screen platereader, Tecan, Salzburg, Austria). The R^2 value was calculated to determine the incubation time showing the best linearity over the standards, and the cell numbers were compared using one-way ANOVA and Tukey's post-tests (Graph Pad Prism). The optimal cell density was 1000 cells per well.

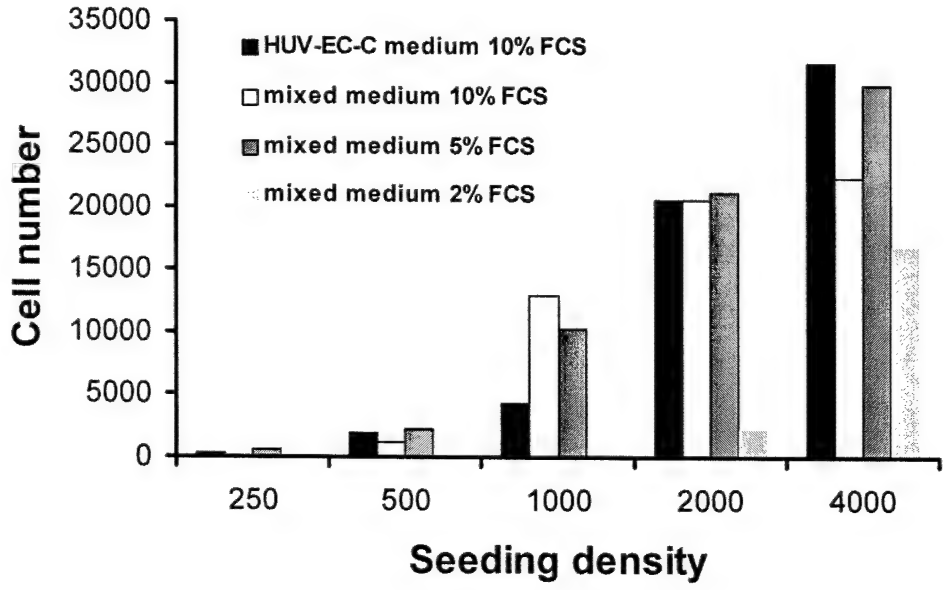
TASK 2A-c

xvi. Optimization of medium for proliferative experiments: As CM contains T medium without FBS, whereas HUV-EC-C cells are grown in F12K and additives (HUV-EC-C medium, Appendix 1), it was necessary to incubate the HUV-EC-C cells in a 50 percent mixture of T medium and HUV-EC-C medium to determine their survival as well as the optimal percentage of FBS for their growth. HUV-EC-C cells were plated in triplicate in 96 well plates at seeding densities of $5x10^2$ to $4x10^3$ cells per well in four sets. Each set of seeding densities was maintained in different medium mixtures as follows:

1) HUV-EC-C medium, 10% FBS
2) 45% HUV-EC-C medium + 45% T medium (Mixed medium) +10% FBS
3) Mixed medium + 5% FBS
4) Mixed medium + 2% FBS

The monitoring and analysis of this experiment were done as described in method xiv, except that the standards were plated at 0, 250, and doubling concentrations thereon to 128000 cells/well. A graph illustrating the cell numbers from this experiment is shown in Figure 5. The optimal seeding cell density in the mixed medium with 5% FBS was also established at 1000 cells per well. The cell proliferation obtained in mixed medium with 5% FBS closely resembled that seen in HUV-EC-C media, with 10% FBS. This mixture will therefore be used in future proliferation experiments. We are now well placed to perform the proliferation studies using the CM and anticipate completing this data set by the end of March, 2003.

Figure 6: Effects of seeding density and media on 7 day proliferation of HUV-EC-C cells



Results:

Based on preliminary data, we have expanded the length of the experiment from 2 days to 7 days to allow any effects of CM on HUV-EC-C cell proliferation to become more apparent.

TASK 3A Role of p53 in bone metastasis *in vivo* using the osseous-CaP bone injection model

Objective:

To determine the take rates of the 6 LNCaP cell lines when injected into the tibia of Severe Combined Immunodeficient (SCID) mice by assaying the host sera for prostate specific antigen (PSA) and by X-ray analysis (Faxitron analysis) of the mice.

Methods:

xvii. Intra-tibial injections: All procedures were performed under sterile conditions in a Class III Cabinet using sterile equipment. Initially, the technique was practised on the left leg of C57BL/6 male mice that had been sacrificed (by overdose with ethrane followed by cervical dislocation) using Trypan Blue. If the dye was contained within the tibia it was scored as a successful injection. The injections were also performed on the right leg. A take rate of 95% on the left and 85% on the right tibia was obtained by L. Perryman. Injection of the left leg was easier to perform, and was used for all following experiments. SCID mice aged from 6 to 8 weeks were anesthetized one at a time by inhalation anesthetic, Isoflurane (5%), carried in oxygen in an induction chamber, and anesthesia was maintained at this concentration for the duration of the procedure using a face mask (around 2 minutes). Cells for injection (2-$4x10^7$cells/mL, for injection of 20µl) were loaded into a 1mL syringe and a 26G needle was attached. The mouse was laid on its back and oriented with the head pointing towards the injecting hand. The ankle joint of its left leg was immobilized between the thumb and forefinger of the left hand (if right handed) and positioned with the knee flexed. The knee was swabbed with 70% alcohol. The cells were injected directly into the proximal epiphysis of the tibia with gentle, unforced drilling, and the needle was removed by the same process in reverse. Analgesia was provided by an intraperitoneal injection of Buprenorphine, (Temgesic, Reskitt and Coleman) at 0.01 mg/kg in a 100µL volume. The mouse was removed from the anesthetic line and allowed to recover.

Three groups of 10 SCID mice were injected intra-tibially with LNCaP, Empty or PC-3 (that are p53 null) cells to determine the take rate of the tumors. The mice were monitored daily. They were weighed and their tibias palpated for tumors twice weekly (according to animal ethics from the Animal Care and Ethics Committee, UNSW, ACEC# 01/102). Every 3-4 weeks, mice injected with LNCaP tumor cell lines were bled for PSA testing. Between 50-100µL serum was diluted with Universal diluent (Roche, Cat #1732277) to 350µL and assayed for PSA using a PSA kit (Roche, Cat #1731262). Eighteen weeks post injection, the mice were sacrificed and X-rayed using a Faxitron (150 VAMAX, Model #MX-20, Auburn, CA, USA; settings 50sec and 20kv). Those mice that scored positive for a leg tumor by autoradiograph were determined to have a final serum PSA level of at least 1.2 ng/mL.

xviii. Histology of bone:
Tibia dissection: A longitudinal incision was made into the skin along the lower half of the hind leg. The tendons were cut just above the ankle, allowing the large muscle to be peeled from the bone. The bone was cut just above the knee joint (distal end of the femur) and also at the distal end of the tibia to allow the fixative to penetrate the bone.

Fixation and decalcification: The legs were placed in 10% Neutral Buffered Formalin for 24-48 hours, then washed in distilled water (dH_20) before placing in 10% formic acid (15-20ml)

in a Falcon tube. The tube was then placed on a rotating platform at an angle (ensuring even decalcification) overnight. The next day, a sample of the formic acid was removed to test for calcium deposits: 3 mL of the formic acid bathing the bone were removed and placed into a fresh tube together with 3mL of saturated ammonium oxalate to determine whether a precipitate forms (this takes about 10 minutes). If a white precipitate is present, the sample requires further decalcification. In this event, the remaining formic acid is removed from the sample and replenished with fresh formic acid. This was repeated daily until no precipitate formed with ammonium oxalate. The sample was then washed 3 times in dH_2O for at least 10min each wash to ensure that all formic acid is removed before placing the samples within the embedding machine.

Staining: Following decalcification, the tissue was paraffin-embedded under standard conditions and stained with hematoxylin and eosin (H & E).

xix. Immunohistochemistry for PSA staining: To demonstrate that the tumor cells identified by the H & E staining were of human origin, immunohistochemical staining for PSA was performed using a polyclonal rabbit, anti-human PSA antibody (A 0562, DAKO, Australia) at 1:4000. The use of this antibody was optimized using human prostate cancer specimens. Rabbit non-specific polyclonal antibodies were used in the same dilution as the primary in adjacent sections to verify the specificity of the observed signals. The sections were incubated overnight at 60°C, then hydrated. Non-specific staining was reduced by incubation with 3% hydrogen peroxide, biotin, avidin and 10% normal goat serum for 5-10min. The sections were incubated with primary or control antibodies for 1 hour, washed and sequentially incubated in goat anti-rabbit antibody (Dako, Carpinteria, CA, USA) at 1:200 for 30 minutes at room temperature, then in Vectastain ABC (Cat # BA-5000, Vector Laboratories, Burlingame, USA) for 30 minutes at room temperature. Color was developed by submerging the slides in freshly prepared diamino benzoate (DAB) (0.05% DAB (D5637, Sigma Chemical Co., St. Louis, MO, USA) for 2min. The slides were counterstained with hematoxylin and Scott blue. The sections were then dehydrated, mounted in 1% Eukitt solution and coverslipped.

Results:

The take rate for the cells is shown in Table 1. Around 50-60% take rate was obtained for LNCaP-parental cells, and 80-90% for LNCaP-Empty, as measured by serum PSA levels or X-ray analysis. Mice were injected with LNCaP-Empty cells after the LNCaP-parental group, and the skills of the investigator could have been improving. For this reason, the work is being repeated. The highest published take rate for LNCaP after intraosseous injections is 75% (Wu *et al* , 1998). The X-rays confirmed, as has been shown by others, that LNCaP cells form mixed/osteoblastic lesions in the bone (Figure 7, see Appendix 5), with numerous bone islands in the injected leg compared to the control. Histological specimens of the LNCaP-parental cells grown in bone were examined after staining with hematoxylin and eosin (Figure 8, see Appendix 6). In addition, staining for PSA was positive in the LNCaP-parental cells grown in bone. The human prostate cancer cell line PC-3, which is null for p53, was used as a monitor of the injection technique as these cells have a reported take rate of 100% take rate after intraosseous injection (Wu *et al*, 1998). The PC-3 injected mice were harvested after 5 weeks and appeared to be healthy. Serum was collected and stored for future assays of bone resorption markers. PC-3 cells do not express PSA. The X-rays at harvest demonstrated a loss of bone when the injected leg was compared to the control leg (see Figure 7, Appendix 5).

Table 2: Take rate of human prostate cancer cell lines in the tibia of SCID mice

Cell line injected	PC-3	LNCaP-parental	LNCaP-Empty
Number of cells injected per mouse	$2x10^5$	$4x10^5$	$2x10^5$
Number of mice with PSA level > 1.2ng/ml	N/A	5 (50%)	9 (90%)
Number of mice with X-ray observable bone remodelling	10	6 (60%)	8 (80%)
Number of mice with tumor cells in the injected tibia by H&E	-	5	5*
Number of mice with tumor cells in the injected tibia by PSA staining	N/A	5	-

n=10 for each cell line

* Only 5 of these mice were processed for histology.

TASK 3B: Intratibial study

Methods:

xx. mRNA extraction from bone: A control experiment was performed to determine the optimal method of RNA extraction from within the bone. The tibiae of a SCID mouse were harvested and all skin and muscle were removed. One was cut into three pieces and placed in Tri-Reagent (Sigma) and incubated for 30 min at room temperature. The second tibia had the ends of the bone removed. A 26G syringe and a 1ml needle were used to drill into the bone marrow cavity and expel the Tri-Reagent 3 times every 10min for half an hour. We found that injecting Tri-Reagent into the bone marrow space provided a greater RNA yield. Furthermore, when tested on an agarose gel, RNA prepared by this method demonstrated minimal degradation of the sample (Figure 9, Appendix 7). This experiment will be repeated in February 2003.

Currently the Faxitron that we are using is located in an area where live mice are not permitted. Messenger RNA (mRNA) degrades with time, so the period between death of the mouse and mRNA extraction must be minimized. We concluded that it would be optimal to X-ray anesthetised mice prior to sacrifice to aid rapid mRNA extraction. We have recently received grants to purchase our own digital Faxitron (The Clive and Vera Ramaciotti Foundation, Cure Cancer Australia Foundation) that will allow us to perform X-ray analysis on anesthetized mice. This should facilitate the harvest of tumors for mRNA analysis. This Faxitron will be available by mid 2003.

A further 10 SCID mice/group have been injected intratibially with LNCaP-parental and LNCaP-Empty cells. The mice were sacrificed at week 18, and the tumors are currently being processed for histology and histomorphometry. Intra-tibial injections of the other cell lines (WT, F134L, M237L, R237H) will be completed by the end of May 2003.

KEY RESEARCH ACCOMPLISHMENTS:

- Performed preliminary experiments to optimize a) cell seeding densities for osteosarcoma cell lines and endothelial cell lines, and b) appropriate media for studies of proliferation and differentiation *in vitro*.
- Collected >1000 mL of conditioned medium (CM) from each of 6 LNCaP cell lines, LNCaP, Empty, Wild type, F134L, M237L and R273H.
- Tested the effects of CM from the six LNCaP lines on the proliferation of three osteosarcoma cell lines, U-2 OS, MG-63 and Saos-2, which mimic osteoblasts at various stages of differentiation *in vitro*. No statistically significant proliferative effects were observed; suggestive effects on U-2 OS and MG-63 proliferation by treatment with 10% F134L CM and on MG-63 proliferation by 50% R273H CM will be re-examined by longer exposure of the osteosarcoma cells to these CM.
- Designed and sequenced primers and optimized RT-PCR for the expression of bone differentiation markers including alkaline phosphatase (ALP), osteocalcin (OCN) and type I collagen (COL) mRNA for examining the effects of CM from the 6 LNCaP cell lines on osteoblast differentiation *in vitro*.
- Incubated 3 osteosarcoma cell lines with CM from the 6 LNCaP cell lines at different doses and time points, and harvested 1350 samples each of cell homogenate in TRI Reagent, and of culture supernatant for RT-PCR and protein analysis.
- Optimized a method for studying matrix mineralization by MC3T3-E1 cells *in vitro*
- Shown that, as opposed to U-2 OS cells, the Saos-2 cells can produce mineralized matrix *in vitro*.
- Established contact with Dr Julian M. W. Quinn from St. Vincent's Institute of Medical Research, Victoria, Australia, who is an expert in the field of osteoclast biology, for assistance in performing studies on the differentiation of osteoclasts by CM from the 6 LNCaP cell lines.
- Created frozen stocks of HUV-EC-C endothelial cells for studies of their proliferation under the influence of CM from the 6 LNCaP cell lines, and optimized the culture conditions for these experiments.
- Established the take rate of LNCaP and Empty cells for forming tumors in the tibia of SCID mice; optimized histological techniques for their examination and immunohistochemical analysis and collected samples for bone histomorphometry.
- Obtained a 100% take rate for PC-3 (p53 null) prostate cancer cells in the tibia of SCID mice, for control studies.

REPORTABLE OUTCOMES:

At this stage of the work, we do not have sufficient data for writing manuscripts, abstracts or patents. However, we have optimized all of the systems, obtained samples for analysis by RT-PCR and ELISA, and obtained the necessary kits for analysis of proteins secreted when osteoblasts are explosed to CM from prostate cancer cell lines expressing wild type p53, overexpressing wild type p53, or expressing mutant p53, and control lines. We are well placed to obtain data very quickly from the next phase of the work.

CONCLUSIONS:

It is difficult to draw conclusions at this phase of the work. The conditioned media from LNCaP parent cells or those stably transfected with Empty vector, over-expressing Wt p53 or mutant p53 have been collected. These do not appear to markedly affect the proliferation of osteoblastic cells *in vitro*. Their effects on differentiation of osteoblasts and osteoclasts *in vitro* are currently being tested. The LNCaP lines have been shown to form tumors when implanted in the tibia of SCID mice, with take rates of ~60% for parental cells and ~80% for those containing the Empty cassette. As we are now fully staffed, we are in an excellent position to answer the questions we have posed. The model for study has been established and should prove suitable for determining the role of *p53* mutations in the establishment and growth of bony metastasis from prostate cancer and could lead to the demonstration of targets for new therapeutic strategies.

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APPENDICES:

Appendix 1: Media ingredients
Appendix 2: Figure 3: RT-PCR cycle titration for collagen primers
Appendix 3: Figure 4: Results of RT-PCR with the osteocalcin primers
Appendix 4: Figure 5: Mineralization of MC3T3-E1 cells
Appendix 5: Figure 7: Faxitron analysis of prostate cancer cell lines established in the tibia of SCID mice
Appendix 6: Figure 8: Photomicrographs of LNCaP-parental tumors after intra-tibial injection of SCID mice.
Appendix 7: Figure 9: Quality of RNA prepared from the tibia of SCID mice

Appendix 1: Media ingredients

"HUV-EC-C- medium": 90% F12K, 10% fetal bovine serum and 30μg/ml of Endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO, USA) and 100μg/ml of Heparin (Sigma).

SOC medium: 2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

"T-medium": DMEM:F-12K, 4:1, supplemented with 5% FBS, 3 g/L sodium bicarbonate, 5 μg/mL insulin, 13.6 pg/mL triiodothyronine, 5 μg/mL transferrin, 0.25 μg/mL biotin, 25 μg/mL adenine.

APPENDIX 2
Figure 3

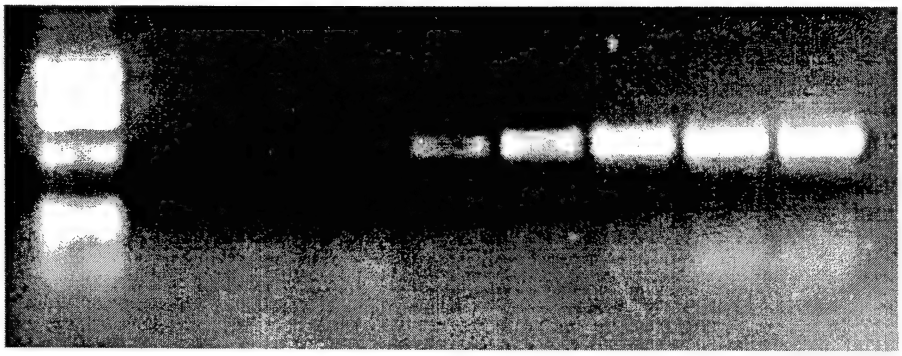
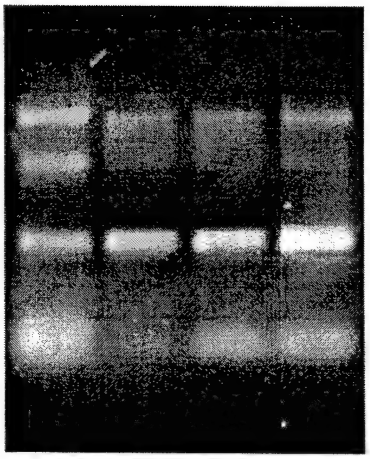


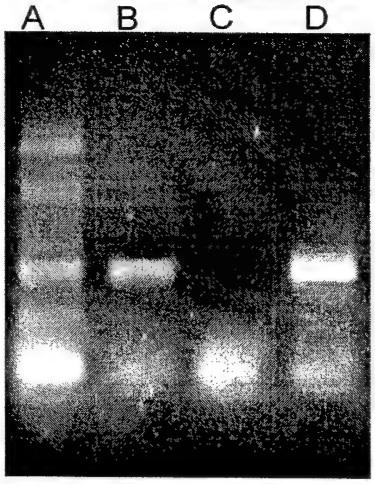
Figure 3: EDAS-scanned agarose gel showing representative RT-PCR cycle titration, using type I collagen primers with *Taq* polymerase and 1.5 mM $MgCl_2$, at 61.5°C. (1) DNA markers; (2) 12 cycles; (3) 15 cycles; (4) 18 cycles; (5) 21 cycles; (6) 24 cycles; (7) 27 cycles; (8) 30 cycles; (9) 33 cycles

APPENDIX 3
Figure 4



OCN (374 bp)

Figure 4a: Results of RT-PCR with the osteocalcin primers under standard conditions using (A) *Taq* polymerase; (B) Platinum PCR Supermix; (C) PCR Supermix; or (D) ReddyMix



OCN (374 bp)

Figure 4b: Results of RT-PCR with the osteocalcin primers, with reduced annealing and extension times: (A) *Taq* polymerase; (B) Platinum PCR Supermix; (C) PCR Supermix; (D) ReddyMix

APPENDIX 4
Figure 5

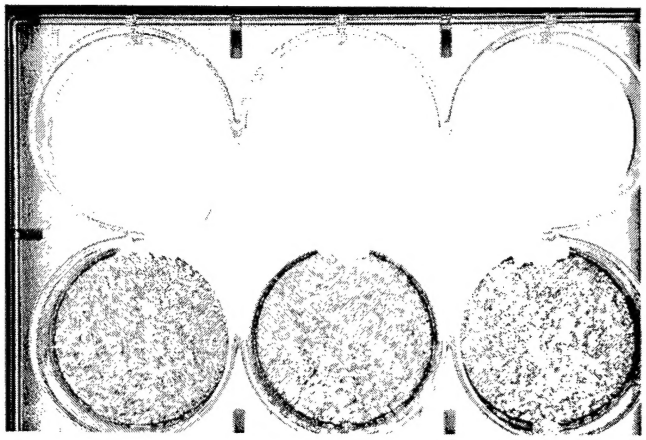


Figure 5: Triplicate wells showing mineralization of MC3T3-E1 cells stained with Alizarin red S. No mineralization occurs in the absence of β-glycerophosphate, (top) which is added one week after seeding (bottom). Addition of dexamethasone increased mineralization further (not shown).

Appendix 5
Figure 7

Intra-tibial injection of LNCaP-parental cells

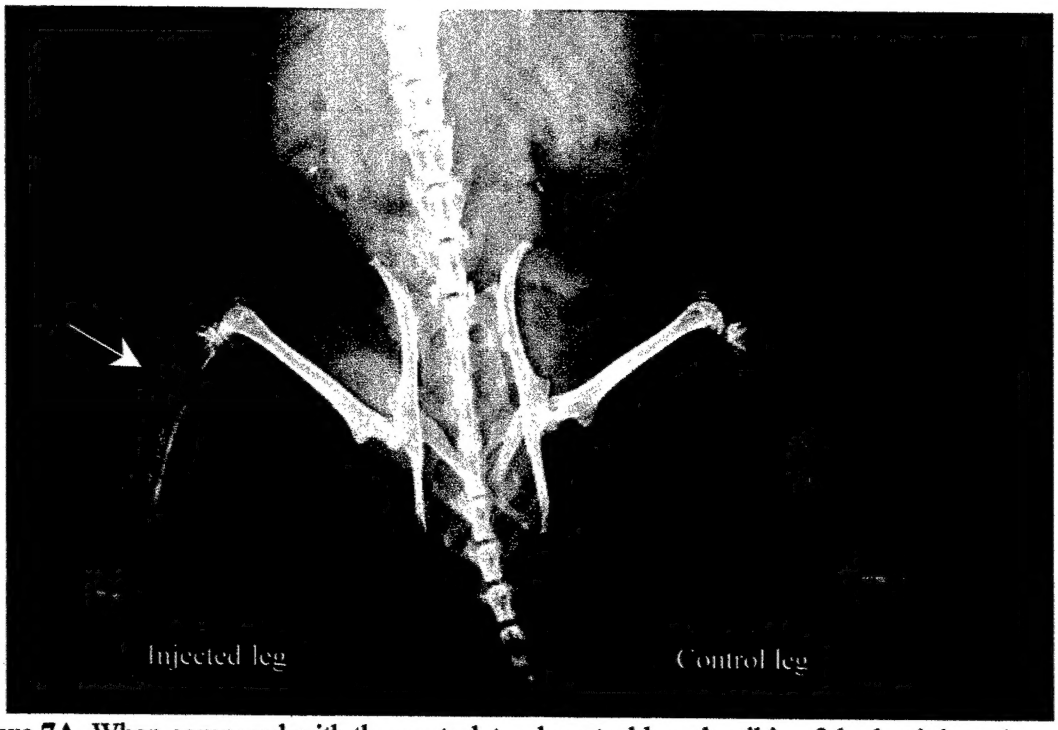


Figure 7A. When compared with the contralateral control leg, the tibia of the leg injected with LNCaP-parental cells exhibited architectural changes after 18 weeks (indicated by the arrow), with some radiographic evidence of a mixed/osteoblastic reaction.

Intra-tibial injection of PC-3 cells

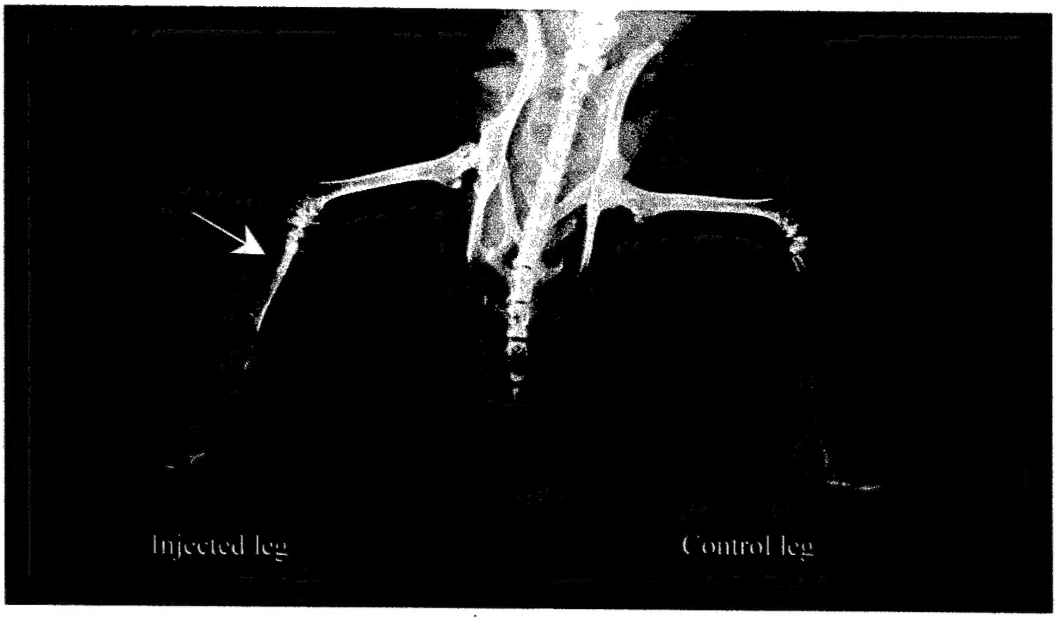


Figure 7B. PC-3 cells produced radiographic evidence of extensive osteolytic degradation in the proximal tibial metaphysis of the injected leg, when compared with the contralateral control leg.

Appendix 6
Figure 8

Figure 8: H&E stained photomicrographs showing intra-tibial prostate cancer tumors of the LNCaP parental line (A, B) grown in the tibia of SCID mice, together with the contralateral control tibia (C, D). A,C were photographed at 100x magnification, and B and D at 250x magnification.

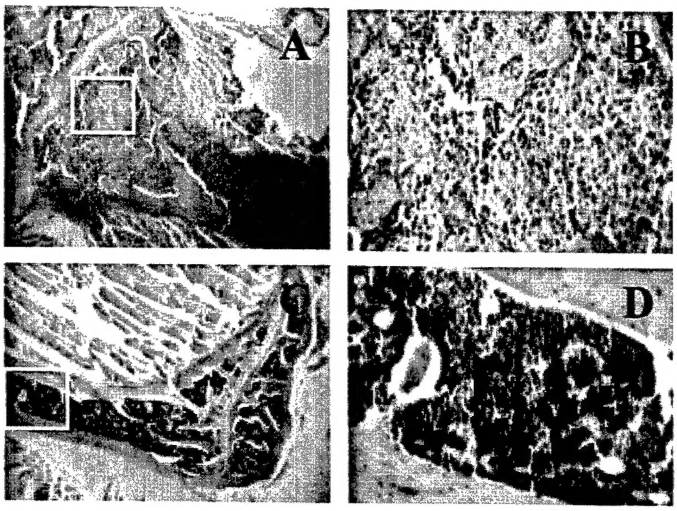
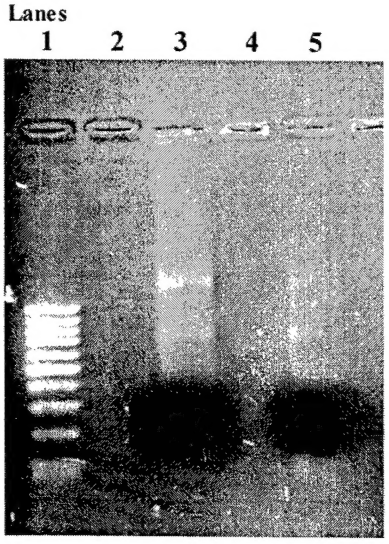


Figure A: This image shows the proximal tibial epiphysis of a tumored leg. Note the abundant numbers of tumor cells within the bone marrow cavity and the rupture of the cortical shaft. There is a decrease in the content of hematopoietic component, possibly due to the volume of tumor cells. This is in contrast to the intact control leg in Figure C. Figures B and D show greater magnification of the white boxed areas.

APPENDIX 7
Figure 9

Figure 9:

Determining the quality of the RNA, extracted from mouse tibia, by gel electrophoresis.



Lane 1: DNA ladder (20 to 100bp).
Lane 2: Empty.
Lane 3: RNA (injecting Tri-reagent into thebone marrow space; RNA extraction procedure).
Lane 4: Empty.
Lane 5: RNA (segments of the tibia incubated in Tri-reagent; RNA extraction procedure).

The bands in lane 3 are brighter than in lane 5; the 28S band in lane 3 is stronger than the 18S band and the lanes do not contain smears. The RNA in lane 3 appears to be of a better quality than that in lane 5.